



**PATENT APPLICATION**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Kelvin G.M. BROCKBANK et al.

Group Art Unit: 1636

Application No.: 09/835,818

Examiner: W. Sandals

Filed: April 17, 2001

Docket No.: 105452

For: CYCLOHEXANEDIOL CRYOPROTECTANT COMPOUNDS

**DECLARATION UNDER 37 C.F.R. §1.131**

Director of the U.S. Patent and Trademark Office  
Washington, D.C. 20231

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We, Kelvin G. M. BROCKBANK, Michael J. TAYLOR, and Lia Hanson CAMPBELL,  
hereby declare and state that:

1. This Declaration is submitted as evidence that the subject matter claimed in Claims 11-19 of the above-identified application was invented by us prior to April 15, 1999, the publication date of WO 99/18169 (PCT/US98/20834).

2. We are the named co-inventors in the above-identified application.

3. Exhibit A is a true redacted copy of excerpts from Quarterly Reports submitted to the Department of Commerce, National Institute of Standards & Technology (NIST).

4. In the copy of the Quarterly Reports attached hereto as Exhibit A, dates and other material which could indicate dates have been masked out. Quarter Report A and Quarter Report B are reports produced in consecutive calendar quarters, both occurring prior to April 15, 1999.

5. Exhibit A describes the design, development, preparation and testing of cryopreservation solutions comprising a combination of compounds, in the United States, before April 15, 1999. The following discussion of Exhibit A is provided:

At page 49, Quarter Report A describes the technical goal to design conditions for long-term tissue and cell storage. A specific objective is to control ice crystal formation using a combination of novel synthetic ice blockers (SIBs), naturally occurring antifreeze compounds, ice nucleators and cryoprotectants. The Report specifies technical milestones, including the control and modulation of ice crystal growth and size, and the evaluation of the effectiveness and cytotoxicity of ice growth-inhibiting formulae.

Beginning at the bottom of page 51, the Exhibit summarizes experimental results that demonstrated that the choice of cryoprotectant-SIB cocktail and the concentration of antifreeze protein, or glycoprotein, each contribute and interact to determine cell survival after freezing. At page 52, the Report contains the results of physical studies on ice crystal formation of a range of potential SIB molecules. As shown in Table 1, solutions containing 1,3-cyclohexanediol (1,3-CHD), 1,4-cyclohexanediol (1,4-CHD), and the combination of 1,3-CHD and 1,4-CHD produced significantly less ice than the control.

At pages 53 and 54, Figures 1 and 2 illustrate the ice crystallization growth rates of various solutions, such as 1,2-CHD, 1,3-CHD, 1,4-CHD, and 1,3-CHO (cyclohexanedione). In summary the Report finds "the most effective compound for controlling ice growth rates from the selection of compounds tested is 1,3-cyclohexanediol." (See page 53).

Following the physical studies, beginning at page 57, Exhibit A reports the cytotoxicity studies. Four SIB compounds, alone, in combination with DMSO, and in combination with other cryoprotectant agents in V49 solution (V49 solution contains 2.75M DMSO, 2.75M formamide, and 2M 1,2 propanediol) were examined for cytotoxicity to J15 and AV5 cells in the absence of freezing. At pages 59 and 61, Figures 6 and 7 summarize the results of these

studies. On the basis of these findings, 1,3-CHD and 1,4-CHD were selected for further study in freezing experiments.

Beginning at page 62, Exhibit A demonstrates the effects of the concentration of 1,3-CHD and 1,4-CHD on the relative viability of AV5 cells frozen in medium containing DMSO. These results are summarized at page 62, Figure 9.

Further experiments, at page 63, demonstrated that combining antifreeze proteins with the optimum concentrations of 1,3-CHD further improved cell viability. This effect is illustrated in at page 64, in Table 5, for AV5 cells frozen in cryopreservation solutions containing various concentrations of the antifreeze protein AFPI, or the antifreeze glycoprotein AFGP. As recognized in Exhibit A, these experiments clearly indicate a synergistic interaction between the antifreeze proteins, cyclohexanediol, DMSO, and the cryoprotectant components of V55 solution (V55 solution includes 0.26M propanediol, 0.37M formamide, and 0.37M DMSO).

Beginning at page 76, the Quarter Report B describes further characterization, development, preparation and testing of cryoprotectant solutions before April 15, 1999. Physical studies of ice crystal growth formation, summarized at page 80, Table 1, demonstrated that 1,3-CHD and 1,4-CHD, either alone or in combination, in a solution containing DMSO and propanediol (DP6), resulted in undetectable or negligible ice crystal formation. Furthermore, as seen in Figure 2, at page 82, 1,3-CHD, alone or in combination with 1,4-CHD, reduced the ice crystallization growth rate of DP6.

Finally, page 93 of Exhibit A describes the results of studies conducted to demonstrate the capability of the developed cryopreservation solutions to protect frozen tissue samples. In on example of this study, jugular vein rings were frozen in various solutions followed by storage in liquid nitrogen. The tissue samples were sectioned and observed under a microscope by a microscopist under double blind conditions. In summary, the cryopreservation solution that

contained 0.5 M 1,3-CHD + 1M DMSO appeared to cause the least amount of cellular damage compared to the other solutions used in the study (see page 94, and Figures 16 and 17).

6. The attached document, which appears as Exhibit B attached to this Declaration, describes the 1,3-cyclohexanediol (1,3-CHD), product number C10-110-9, supplied by Sigma-Aldrich Co., and used throughout the experiments described in Exhibit A. The Sigma compound includes both cis and trans forms of 1,3-CHD.

7. Thus, Exhibits A and B describe a cryopreservation composition comprising at least one cyclohexanediol compound and at least one additional cryoprotectant compound.

8. Exhibits A and B also describe a cryopreservation composition comprising at least one cyclohexanediol compound and at least one additional cryoprotectant compound wherein:

- the at least one cyclohexandiol compound is selected from the group consisting of the cis form of 1,3-cyclohexanediol, the trans form of 1,3-cyclohexanediol, the cis form of 1,4-cyclohexanediol, the trans form of 1,4-cyclohexanediol, and racemic mixtures thereof.
- the cyclohexanediol compound is present in the cryopreservation composition in an amount of from 0.5 to 2.0 M.
- the at least one least one additional cryoprotectant compound is selected from the group consisting of acetamide, agarose, alginate, l-analine, albumin, ammonium acetate, butanediol, chondroitin sulfate, chloroform, choline, dextrans, diethylene glycol, dimethyl acetamide, dimethyl formamide, dimethyl sulfoxide (DMSO), erythritol, ethanol, ethylene glycol, formamide, glucose, glycerol,  $\alpha$ -glycerophosphate, glycerol monoacetate, glycine, hydroxyethyl starch, inositol, lactose, magnesium chloride, magnesium sulfate, maltose, mannitol, mannose, methanol, methyl acetamide, methylformamide, methyl ureas, phenol, pluronic polyols, polyethylene glycol, polyvinylpyrrolidone, proline, propylene glycol, pyridine N-oxide, ribose, serine, sodium bromide, sodium chloride, sodium iodide, sodium nitrate, sodium sulfate, sorbitol, sucrose, trehalose, triethylene glycol, trimethylamine acetate, urea, valine and xylose.

- the at least one additional cryoprotectant compound is present in the cryopreservation composition in an amount of from 0.1 to 10.0 M.
- the cryopreservation composition further contains at least one anti-freeze protein.
- the cryopreservation composition further contains at least one anti-freeze protein present in the cryopreservation composition in an amount of from 0.1 to 1 mg/mL of the cryopreservation composition.
- the cryopreservation composition further contains at least one anti-freeze glycoprotein.
- the cryopreservation composition further contains at least one anti-freeze glycoprotein present in the cryopreservation composition in an amount of from 0.1 to 1 mg/mL of the cryopreservation composition.

9. Exhibits A and B describe an invention conceived and reduced to practice by us in the United States prior to April 15, 1999. This invention is claimed in the above-identified application.

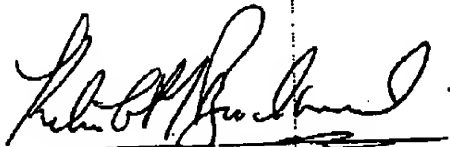
10. Prior to April 15, 1999, we or those under our direct control and supervision, carried out a reduction to practice in the United States of the invention described in Exhibits A and B and thereby provided a cryopreservation composition, as described in paragraphs 5-8 herein.

11. In the United States, prior to April 15, 1999, we made and tested physical embodiments of the cryopreservation composition, as described in paragraphs 5-8 herein.

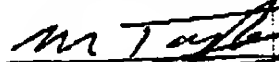
12. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Attachment:  
Exhibit A  
Exhibit B

Date: Jan 21<sup>st</sup> 2003

  
Kelvin G. M. BROCKBANK

Date: January 21<sup>st</sup> 2003

  
Michael J. TAYLOR

Date: January 21<sup>st</sup> 2003

  
Lia Hanson CAMPBELL

# Exhibit

## A

## **██████████ QUARTER REPORT A**

Date of Summary: ██████████

### **Project Objectives:**

The overall technical goal of this project is the definition and design of conditions for long-term tissue and cell storage that will make it possible for tissue engineered devices to be available both in the United States and worldwide, regardless of environmental conditions.

Specific objectives are:

1. Control of ice crystal formation using a combination of novel synthetic ice blockers (SIBs), naturally occurring antifreeze compounds, ice nucleators and cryoprotectants.
2. Avoidance of ice formation by vitrification.

### **Technical Milestones:**

1. Demonstration of non-toxic ice crystal growth control by modulation of ice crystal size in solutions and tissues.
  - (a) Evaluation of the effectiveness of ice growth-inhibiting formulae using physical study methods.
  - (b) Evaluation of the toxicity of ice growth-inhibiting formulae.
  - (c) Evaluation of the results of freezing and thawing vein rings in media designed to contain ice crystal size using *in vitro* and *in vivo* function tests.
2. Creation of new SIB molecular designs with high potential for minimizing ice crystal formation and development of synthesis strategies where necessary.
3. Evaluation of rewarming methods.
  - (a) Identification of the best technique for warming to be deployed for future research and development.
  - (b) Determination of whether or not slower rate resonance radio frequency warming or convection warming is possible with retention of viability in the presence of exogenous agents such as ice nucleating agents.
4. Development of preservation methods to be applied to single cell suspensions and small cell aggregates.
5. Completion of assessment of current first choice baseline vitrification solution consisting of 3.10M DMSO, 3.10M formamide and 2.21M 1,2-propanediol in Euro-Collins Solution. Establishment of an optimal formulation using these 3 reagents in the vascular model.

## **Technical Progress and Impact:**

### **Executive Summary**

The ■ quarter has yielded exciting results from both of the major research pathways. The avoidance of ice by the vitrification pathway has resulted in preserved vessel segments which retain greater than 80% *in vitro* smooth muscle function and *in vivo* patency rates equivalent to fresh autograft controls. Potential improvements in the vitrification process, which may result in a decreased risk of cryoprotectant cytotoxicity have been identified in the physical studies of ice formation in bulk samples. The earlier results in the ice control pathway experiments using *in vitro* adherent cell models have been confirmed and expanded for statistical analysis. These experiments have demonstrated that the choice of cryoprotectant-synthetic ice blocker (SIB) cocktail and the concentration of antifreeze protein, or glycoprotein, each contribute and interact to determine cell survival after freezing ( $P < 0.01$ , 2-way ANOVA). Freeze substitution experiments have demonstrated modulation of ice forms within tissues that depend upon the combinations of cryoprotectants, antifreeze compounds and SIBs. We anticipate further important developments in vitrification and ice control methods in the studies planned for the coming year.

### ***Facility & Personnel***

*Current Personnel.*

Personnel	Role	Effort on Project	Start Date
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Milestone Contribution (1a): "Evaluation of the effectiveness of ice growth-inhibiting formulae using physical study methods" and (2) "Creation of new SIB molecular designs with high potential for minimizing ice crystal formation and development of synthesis strategies where necessary".

### **Ice Crystal Formation**

During this phase of the physical studies on ice formation we have extended the preliminary screening work outlined previously by replicating measurements in the key experimental groups. These are defined as those in which control of ice crystallization and growth was indicated to be measurably different from control groups. For reference, two cryoprotectant mixtures were selected as controls. The first, VS55 is our reference vitrification

solution, which contains a total concentration of cryoprotectants = 8.4 molar, and is known to vitrify completely when cooled at slow rates ( $>1^{\circ}\text{C}/\text{min}$ ). By contrast, a diluted version of VS55, containing the same proportional mix of CPAs at a total concentration of 7.5M (V49), freezes readily when cooled slowly ( $<3^{\circ}\text{C}/\text{min}$ ) below  $-34^{\circ}\text{C}$ . Table 1 shows mean ( $\pm\text{SEM}$ ) data for ice crystallization measurements derived from bulk freezing experiments using the techniques described previously. The relative activity of a range of potential synthetic ice blocker (SIB) molecules was evaluated by incorporating them at a concentration of 6% in the V49 solution. It was observed that the V49 solution without added solutes froze extensively under these conditions producing an indefinite number of ice crystals that occupied the entire area of the freezing chamber. In marked contrast the VS55 solution consistently showed the formation of a number of very small ice crystals when cooled to  $-100^{\circ}\text{C}$  at  $\sim 1.5^{\circ}\text{C}/\text{min}$  in our system, but this restricted ice formation occupied only  $\sim 1\%$  of the total area of the bulk sample. These conditions of cooling thus provided a critical evaluation of the tendency to freeze in bulk samples of solutions containing high concentrations of cryoprotectants. Table 1 shows that all solutions, with the exception of V49 + sucrose, produced significantly less ( $p < 0.001$ ) ice than the V49 solution alone. Measurements of ice crystal number and the total area occupied by ice should not be considered independently because some solutions such as V49 + sucrose appear to produce a very high percentage of ice from a few nucleation sites, compared with the VS55 control vitrification medium that yields only 1.2% total ice from  $46 \pm 6$  nucleation sites.

**Table 1: Bulk Phase Ice Crystallization Measurements from Image Analysis**

Cryoprotectant Solution	Ice Crystal #	Total Area %
V49 (7.5M CPAs)	Indefinite	100
VS55 (8.4M CPAs)	$45.7 \pm 5.8$ (27)	$1.21 \pm 0.12$ (27)
V49 + NaCl	$609 \pm 104$ (3)	$22.2 \pm 1.0$ (3)
V49 + Sucrose	$5.3 \pm 2$ (3)	$99.8 \pm 0.1$ (3)
V49 + 1,2-Cyclohexanedione (1,2 CHO)	$107 \pm 23$ (2)	$3.6 \pm 0.2$ (2)
V49 + 1,3-Cyclohexanedione (1,3 CHO)	$12.5 \pm 8.5$ (2)	$0.32 \pm 0.27$ (2)
V49 + 1,2-Cyclohexanediol (1,2 CHD)	$108 \pm 55$ (3)	$2.16 \pm 0.7$ (3)
V49 + 1,3-Cyclohexanediol (1,3 CHD)	$173 \pm 76$ (4)	$2.27 \pm 0.7$ (4)
V49 + 1,4-Cyclohexanediol (1,4 CHD)	$107 \pm 66$ (5)	$1.68 \pm 0.55$ (5)
V49 + 1,3 and 1,4-Cyclohexanediol	$31.3 \pm 8.1$ (3)	$0.54 \pm 0.07$ (3)

Values are the Mean  $\pm$  SEM (n).

One interpretation of this is that slow cooling of a vitrification medium under these conditions can result in the formation of ice nuclei but these do not grow during the cooling phase. By contrast, fewer nucleation sites in V49 + sucrose appear to grow more rapidly during cooling such that almost complete freezing has occurred by  $-100^{\circ}\text{C}$ .

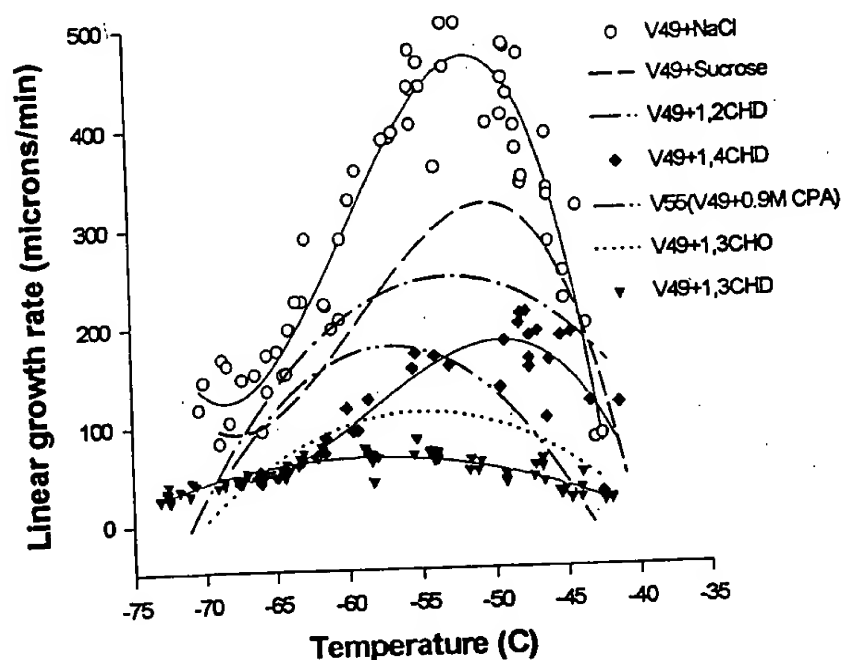


Fig.1 Kinetics of ice crystallization growth in VS55 solution compared with V49 cryoprotectant solution containing 6%(w/v) of various solutes including synthetic ice blocker molecules. The lines represent 3<sup>rd</sup> order regression analysis of the data in each group.

It is noteworthy that all of the SIB compounds were highly effective in reducing the amount of ice formation and resulted in significantly less ice than either the V49 solution alone, or V49 + sucrose ( $p < 0.001$ ). Clearly, the SIB molecules used individually, or in combination, are more effective than the same percentage concentration of other solutes such as sodium chloride, or sucrose used as alternative solutes having a high colligative function and, in the case of

sucrose, also regarded as a good glass forming agent.

### Ice Crystallization Growth Rates

The measurement of ice crystal growth kinetics is of great importance as we have indicated above and in previous reports. Fig.1 summarizes the linear ice growth rate measurements in V49 solution containing various solutes. At this point the most effective compound for controlling ice growth rates from the selection of compounds tested is 1,3-cyclohexanediol (1,3-CHD). The marked effect of this compound on the kinetics of ice crystallization in V49 solution is shown in more detail in Fig.2. The rate of ice growth in a cryoprotectant solution such as V49 was observed to increase initially as cooling proceeded below the freezing point, to a maximum rate followed by a decline as temperature was reduced further. Fig.2 shows that the steep increase in ice crystal growth rate in V49 is suppressed in the VS55 vitrification solution that contains an additional 0.9 moles/l of the cryoprotectant mix. Moreover, Fig. 2A shows that the addition of 6% (0.5M) 1,3-CHD reduces the rate of ice crystal

growth to less than  $50\mu\text{m}/\text{min}$  over the entire temperature range between  $-40^\circ$  and  $-75^\circ\text{C}$ . This represents a four-fold decrease over the maximum rate in VS55 in the range of  $-60^\circ\text{C}$  and an impressive 20-fold decrease over the peak rate in V49 in the region of  $-50^\circ\text{C}$ . It is also important to note that this effect was achieved using a low cooling rate of  $\sim 1.5^\circ\text{C}/\text{min}$  which we anticipate to be a practical cooling rate for the cryopreservation of sizeable pieces of tissue.

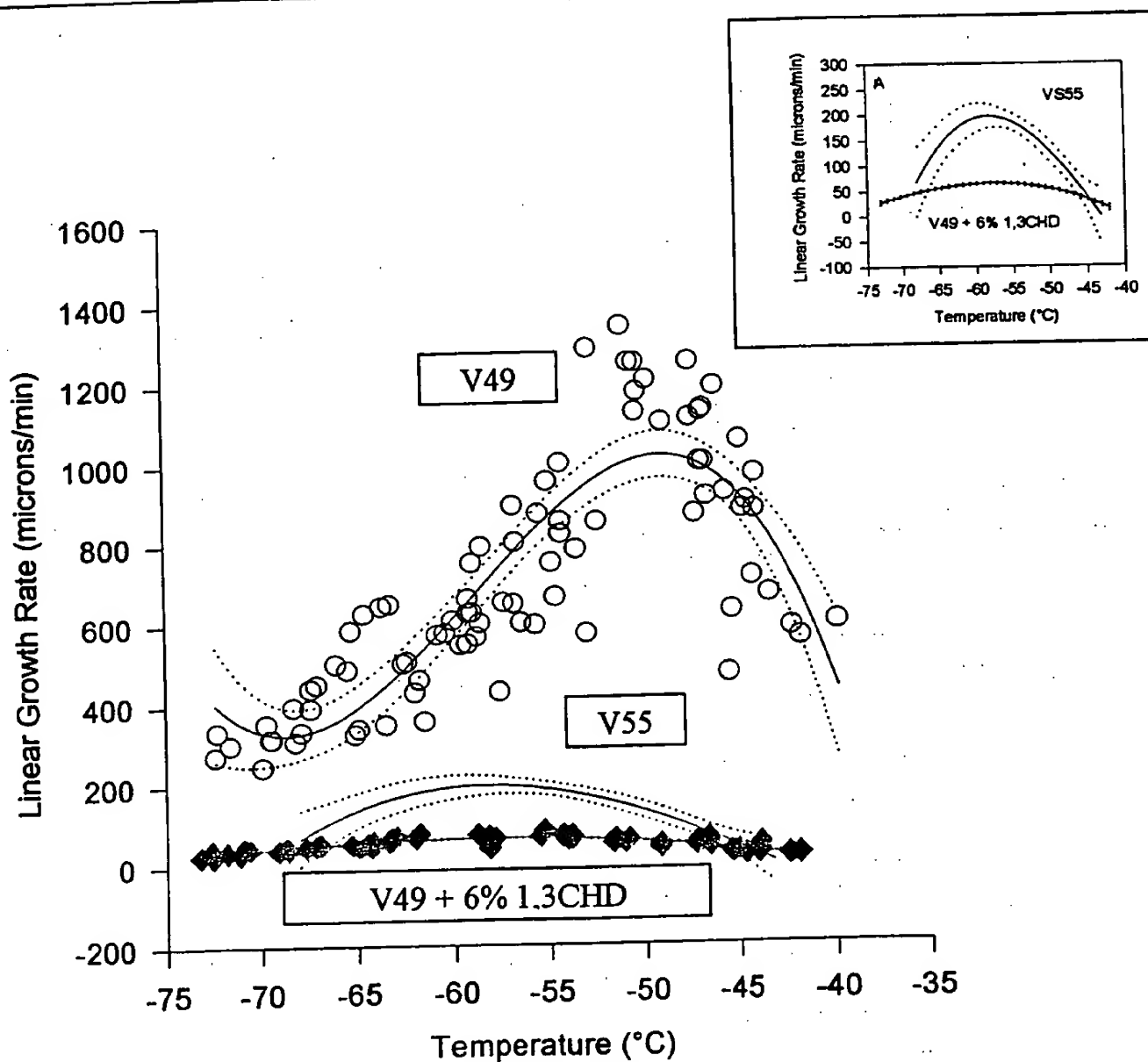


Fig.2. Kinetics of linear ice crystallization growth in vitrification solutions as a function of temperature. Solid lines represent the 3<sup>rd</sup> order regression curves fitted to the data with 95% confidence limits shown as dotted lines. The inset graph (A) shows the regression lines for V55 and V49 + 6% 1,3CHD on an amplified scale.

## Alternative Vitrification Solutions

During this quarter, we have continued to evaluate some alternative vitrification solutions designed to vitrify with a lower total concentration of cryoprotectants than V55 in order to minimize cytotoxicity. We have now extended the preliminary studies reported in the quarter report using solutions containing various proportions of DMSO and propanediol, but without any formamide, which we have shown to be a more cytotoxic than either of the other two CPAs (see cytotoxicity studies described in a subsequent section of this report). Specifically, we selected solutions F1 (comprising DMSO (3.0M) and propanediol (3.2M)), and F2 (containing 3.0M DMSO and 3.4M propanediol) from the pilot studies (Table 3 of the Q3 report). As shown in Fig. 3A, solution F2, having a total CPA concentration of 6.4M, was more effective at suppressing the rate of ice crystal growth compared with the F1 solution (total CPA = 6.2M).

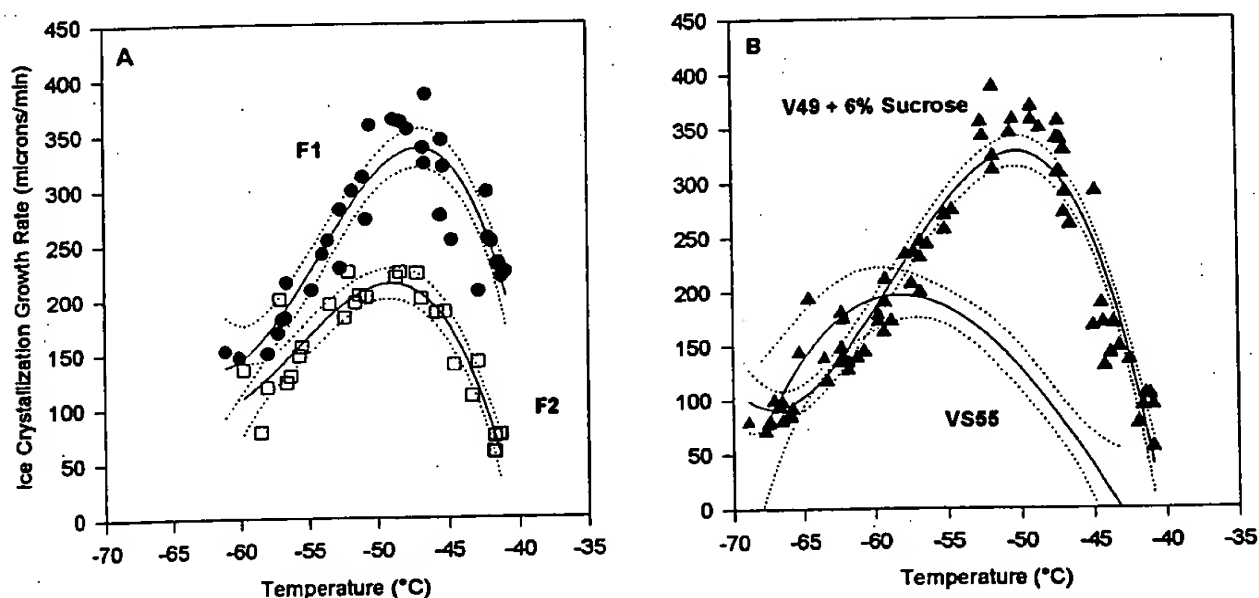


Fig. 3, Ice crystal growth kinetics for new alternative vitrification solutions compared with the V55 solution. The graphs show 3<sup>rd</sup> order regression plots (solid lines) fitted to the data in each case with 95% confidence limits (dotted line). For clarity the control data for V55 solution is shown as the regression plot only.

For comparison, the ice growth kinetic plots for VS55 and V49 + Sucrose and shown in Fig. 3B. From these curves it can be seen that the rates of ice growth in the F1 solution with a total CPA concentration of 6.2 moles/l, is similar to the V49 + sucrose solution having a total CPA concentration = 7.68M. Moreover, the F2 solution (total CPA = 6.4M) was seen to suppress the maximum rate of crystal growth to levels comparable with the standard VS55 vitrification solution that contains 8.4 moles/l of cryoprotectants. Clearly, from a biocompatibility point of view, it would be preferable to use the F2 solution, which has a significantly lower total concentration of CPAs, compared with VS55 if the physical properties of ice-control during

cooling are similar. It should be noted, however, that the temperature at which the maximum rate of crystal growth is observed, was different in the F1 and F2 solutions compared with VS55. This is illustrated more clearly in Fig 4 where the regression curves for F2 and VS55 are

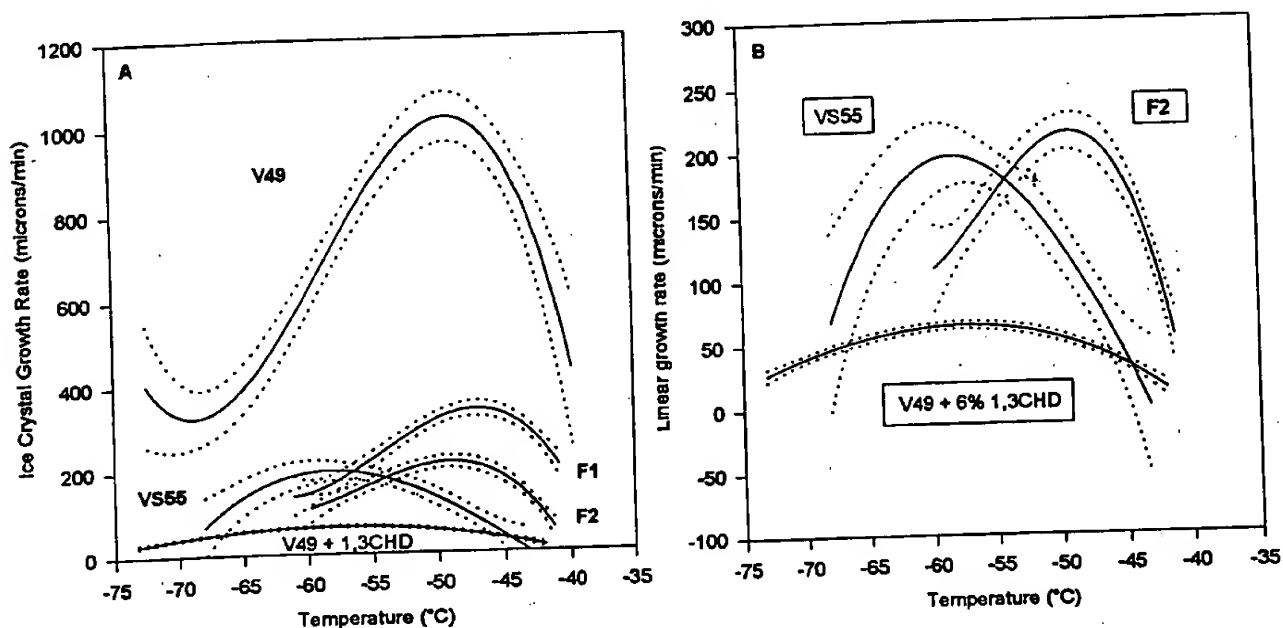


Fig. 4. Kinetics of ice crystallization growth in V49 solution, with or without 6% 1,3CHD, compared with the control VS55 vitrification solution and the new formulations, F1 and F2. Panel B shows the same curves for VS55, F2 and V49 + 1,3CHD on an expanded scale. The graphs show the 3<sup>rd</sup> order regression curves with 95% confidence limits in each case and the data points are omitted for clarity.

compared directly. It can be seen in Fig. 4B that the ice growth rate curves for these two solutions have optima at two distinctly different temperatures separated by about 10 degrees. More importantly, the curve for V49 + 1,3CHD discussed earlier, has been included in Fig. 4 to illustrate the robust effect of the SIB compound in flattening the V49 curve to low rates of growth over the entire temperature range from -40° to -75°C. The effect of adding SIB compounds to these new solutions such as F1 and F2 will be evaluated during the next quarter. We anticipate that in the presence of SIBs it will be possible to effectively control ice growth in solutions containing markedly lower concentrations of cryoprotectants than our baseline vitrification solution (VS55).

### Thermal Hysteresis

The planned experiments measuring thermal hysteresis in solutions containing mixtures of antifreeze proteins and synthetic ice blocking molecules had to be held in abeyance during this quarter due to the fact that the Clifton nanoliter osmometer had to be returned to the manufacturer for repairs. The instrument has now been recommissioned and calibrated ready for resuming these studies during the next quarter.

Milestone contribution: (1b) "Evaluation of the toxicity of ice growth-inhibiting formulae" and (4) "Development of preservation methods to be applied to single cell suspensions and small cell aggregates"

### *Cytotoxicity in the absence of freezing*

In accordance with the plans outlined in previous reports we have extended the studies evaluating cytotoxicity of various cryoprotectant cocktails to a number of cell lines relating to our focus on the preservation of cardiovascular tissues.

#### A. Primary venous smooth muscle cells

Studies initiated in the last quarter using primary smooth muscle cells (J15) from rabbit jugular veins have proceeded more slowly than anticipated. This was due principally to the fact that these primary cells undergo periods of very slow growth that limits the rate at which sufficient numbers of cells are attained for the planned experiments.

**Table 2: Experimental design for cytotoxicity using J15 primary smooth muscle cells**

A. VS55 & components in EuroCollins vehicle solution			
CPA	Concentration range	N (rep x exp)	
DMSO	0-5M	5 rep x 3 exp.	
Formamide	0-5M	5 rep x 3 exp	
Propanediol	0-5M	5 rep x 3 exp	
VS55	0-8.4M	5 rep x 3 exp	
B. Synthetic Ice Blockers (SIB) molecules in EuroCollins solution			
SIB	Concentration range	N (rep x exp)	
1,3 CHD	0-1M	5 rep x 3 exp	
1,4 CHD	0-1M	5 rep x 3 exp	
1,2 CHD	0-1M	5 rep x 3 exp	
1,3 CHO	0-1M	5 rep x 3 exp	
C. SIB molecules combined with other CPAs in EuroCollins solution			
SIB	Concentration range	CPA	N (rep x exp)
1,3 CHD	0-1M	1M DMSO	4 rep x 1 exp.
		1M V49 (7.5M)	4 rep x 1 exp.
1,4 CHD	0-1M	1M DMSO	4 rep x 1 exp.
		1M V49 (7.5M)	4 rep x 1 exp.
1,2 CHD	0-1M	1M DMSO	4 rep x 1 exp.
		V49 (7.5M)	4 rep x 1 exp.
1,3 CHO	0-1M	1M DMSO	4 rep x 1 exp.
		V49 (7.5M)	4 rep x 1 exp.

Table 2 shows the outline of the experimental design for the studies completed to date for evaluating the tolerance of J15 cells to increasing concentrations of the cryoprotectants that constitute our baseline vitrification solution VS55. The design was similar to that undertaken previously using the A10 smooth muscle cell line. As before cytotoxicity was evaluated using the Alamar Blue assay and the results are summarized in Fig 5.

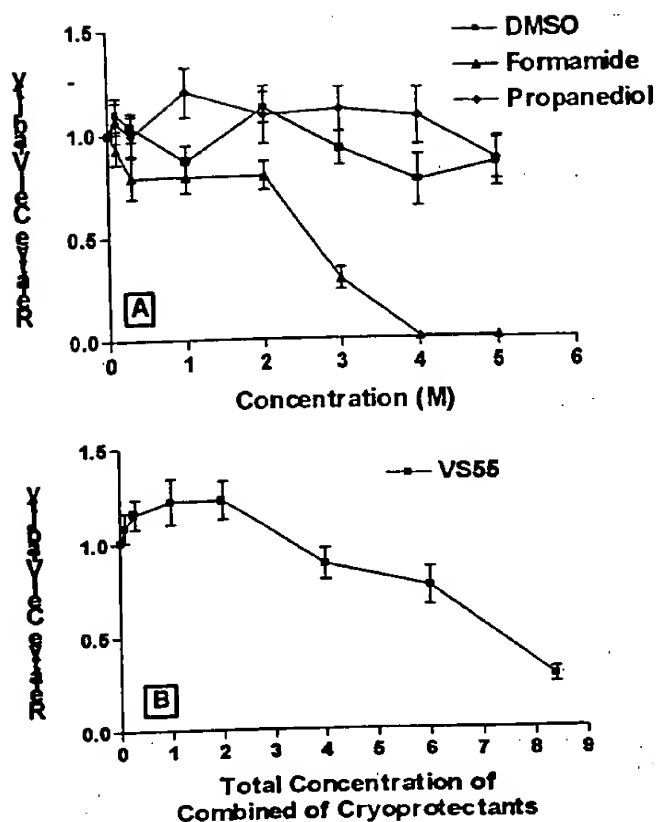


Fig 5. Relative cell viability of J15 cells after exposure to increasing concentrations of the cryoprotectants used in the formulation of VS55 vitrification solution.

Fig 5A shows that each of the individual cryoprotectants was well tolerated up to a concentration of 2.0M. The mean viability of cells exposed to formamide was consistently lower than for the other CPAs and viability declined precipitously when the concentration of formamide exceeded 2M. In contrast, a high level of viability was maintained after exposure to both DMSO and propanediol at concentrations up to 5M. A similar dependence on total CPA concentration was observed when the J15 cells were exposed to the combined CPAs in VS55 solution. Fig 5B shows that viability declined when the total concentration of the component CPAs exceeded 2M. In view of the results with the individual CPAs (Fig 5A), it is likely that the toxicity of formamide predominates in contributing to the observed toxicity of VS55 at concentrations >2M.

The general responses of the J15 cells to CPA toxicity was similar to that observed for the A10 cells, but the J15 cells demonstrated an increased cytotoxic sensitivity, especially to formamide.

The next phase of the study with J15 cells was to evaluate their tolerance to the four SIBs that had been selected for

screening using the A10 cell line. For these experiments a narrower range of concentrations of the SIB compounds was tested in order to judge more precisely the acceptable concentrations that might be used safely in the cryopreservation protocols. Fig. 6 shows that the averaged relative viability curves for all four SIBs (1,2-CHD; 1,3-CHD; 1,4-CHD and 1,3-CHO) overlap at concentrations up to 0.50M; thereafter, the curves diverge demonstrating a clear differential sensitivity of these cells to higher concentrations of the individual compounds. Additional experiments have recently been initiated in which these SIB compounds are combined with either 1M DMSO, or the combined cryoprotectants in V49, as outlined in Table 2 above. The outcome of these studies will be reported in the next report.

## B. Primary heart valve myofibroblasts

During the period of this report a new study using myofibroblast cells was undertaken to parallel the studies on *in vitro* cytotoxicity testing using the A10 and J15 smooth muscle cells. In addition, freezing experiments were undertaken for evaluation of the cryoprotection afforded these cells by various combinations of the CPAs and ice-control molecules.

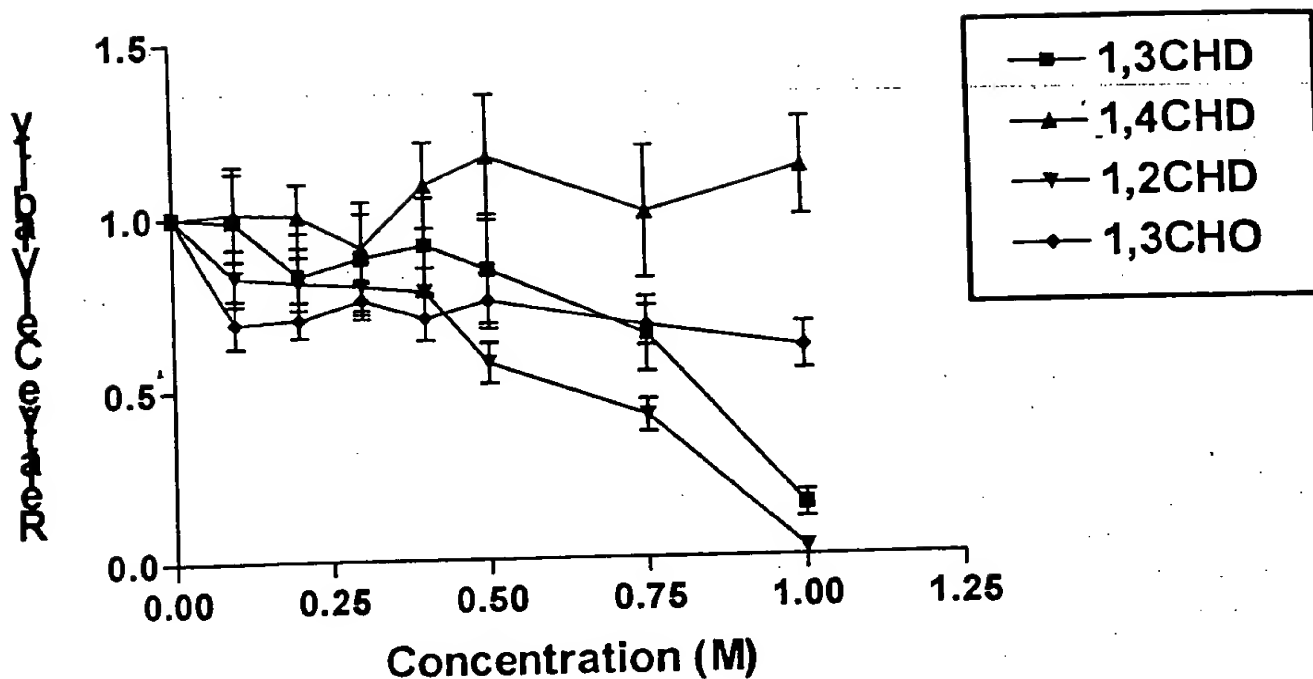


Fig 6. Relative Cell Viability of J15 cells after exposure to selected synthetic ice blocker (SIB) compounds. J15 cells were plated at  $1 \times 10^4$  cells/well. The next day, cell viability was assessed at 37°C using Alamar blue after exposure to varying concentrations of the SIBs at 0°C for 10mins. The data is presented as the means ( $\pm$  SEM) of 15 replicates.

## Methods

In brief, a primary culture of myofibroblast cells (AV5) from porcine heart valve leaflets was established and banked using a standard cryopreservation technique for future use. For the cytotoxicity assay AV5 cells were plated in a 96-well plate at a concentration of  $1 \times 10^4$  cells/well in 50  $\mu$ l of culture medium (DMEM +10%FCS). The next day cells were cooled in preparation for exposure to cryoprotectant solutions by placing the plate on ice. A range of cryoprotective additives (CPA) was investigated according to the schedule outlined in Table 3. The CPA mixtures were prepared in EuroCollins solution as the carrier medium and added to the cells for 10 minutes before being removed using a stepwise washing technique with a solution containing mannitol (0.5M). Cells were assayed for viability using the Alamar Blue fluorescent indicator that measures cell metabolic activity in the culture plate at 37°C. Cells were also assayed in the same way after freezing and thawing. In these experiments cells were plated at density of  $2.5 \times 10^4$  cells/well and the CPA combinations were added in the same way as in the cytotoxicity studies. For freezing, the plates were cooled at 1°C/min in a program-controlled-rate freezer to -80°C before being stored in the gas phase of liquid nitrogen at  $\sim -130^\circ\text{C}$ . For thawing, the plate was placed at room temperature until the temperature of the samples reached  $-20^\circ\text{C}$ , whereupon the plate was transferred to 37°C for the initial step of the CPA dilution regimen. The remaining steps were carried out under hypothermic conditions with the plate on ice. Finally, the thawed-washed cells were rewarmed to 37°C and assayed for viability using the Alamar Blue assay.

## Results

The experimental design for the cytotoxicity studies was to examine the tolerance of AV5 cells to a range of concentrations of the cryoprotectants that comprise the VS55 vitrification solution; namely, dimethyl sulfoxide (DMSO), propanediol, and formamide. This evaluation was carried out as described previously for other cell types and as outlined in Table 3. DMSO and propanediol were well tolerated up to concentrations of 2-3M, but formamide clearly produced a noticeable decrease in viability when used at concentrations >1M. Interestingly, these cells tolerated the combined CPAs up to a total CPA concentration of 2 molar before a decrease in cell viability was detected in comparison with control cells that were maintained for the same length of time in EuroCollins solution without any added CPA.

Table 3. Outline of cytotoxicity experiments with AV5 cells

A. V55 & components in Euro-Collins			
CPA	Concentration range	N (rep x exp)	
DMSO	0-5M	4 rep x 4 exp.	
Formamide	0-5M	4 rep x 4 exp	
Propanediol	0-5M	4 rep x 4 exp	
V55	0-8.4M	4 rep x 4 exp	
B. Synthetic Ice-Blockers (SIBs) in Euro-Collins			
SIB	Concentration range	N (rep x exp)	
1,3 Cyclohexanediol(CHD)	0-2M	4 rep x 4 exp.	
1,4 Cyclohexanediol	0-2M	4 rep x 4 exp	
1,2 Cyclohexanediol	0-2M	4 rep x 4 exp.	
1,3 Cyclohexanedione(CHO)	0-2M	4 rep x 4 exp	
C. SIBs with other CPAs in Euro-Collins			
SIB	Concentration range	CPA	N (rep x exp)
1,3 CHD	0-2M	1M DMSO	4 rep x 4 exp
		V49 (7.5M)	4 rep x 4 exp
1,4 CHD	0-2M	1M DMSO	4 rep x 4 exp
		V49 (7.5M)	4 rep x 4 exp
1,2 CHD	0-2M	1M DMSO	4 rep x 4 exp
		V49 (7.5M)	4 rep x 4 exp
1,3 CHO	0-2M	1M DMSO	4 rep x 4 exp
		V49 (7.5M)	4 rep x 4 exp

Using the same rationale as that used in the design of the studies with other cell types, the next stage of this study was to examine the biocompatibility of the ice-control molecules selected from the physical studies. As shown in Table 3, four SIB molecules were selected for further study. Cytotoxicity to AV5 cells was examined for the SIB compounds alone, and in combination with DMSO, or the combined CPAs of V49 solution containing a total CPA concentration of 7.5M. It should be noted that DMSO was selected for study in the single CPA studies because it was shown to be well tolerated over the entire concentration range studied (0-

5M), and also because it is the CPA used currently in clinical practice for cryopreservation of heart valves. The cell viability indices with respect to the SIB additive are summarized in Fig. 7. It is clear that the cytotoxicity of these compounds varies as a function of concentration and type. Both 1,3-CHD and 1,4-CHD were well tolerated up to concentrations of 2 molar, but 1,3-CHO was clearly toxic at this concentration and 1,2-CHD at concentrations as low as 0.5M. Similar responses were observed in the presence of other CPAs with 1,3-CHD and 1,4-CHD showing significantly less cytotoxicity compared with either 1,2-CHD or 1,3-CHO. All SIB compounds, except 1,3-CHD, demonstrated measurable toxicity when used at a concentration above 0.25M in the presence of V49.

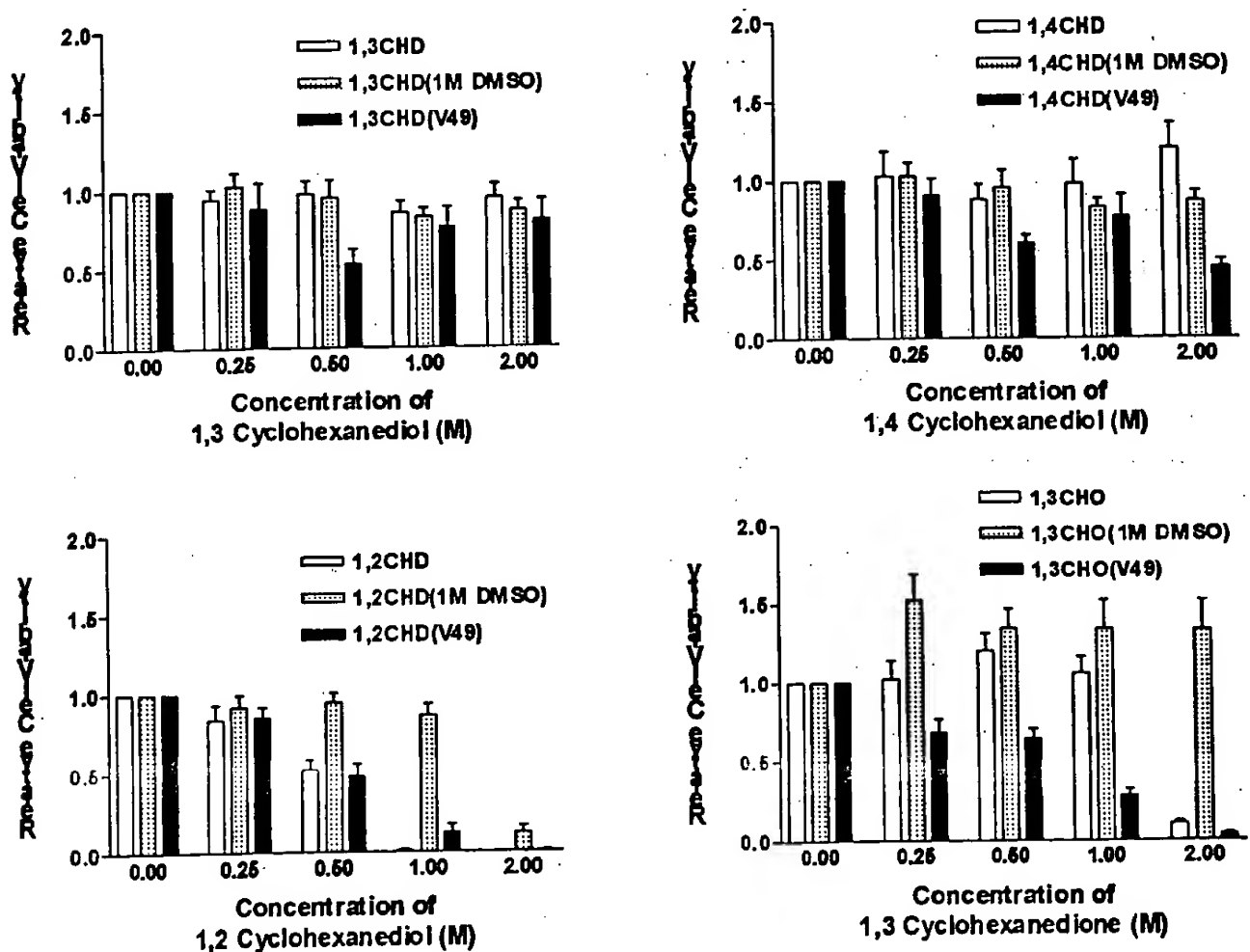


Fig.7. Relative cell viability of AV5 cells after exposure to synthetic ice blockers alone and in combination with either DMSO (1M), or V49 (7.5M total concentration of CPAs).

On the basis of these findings, 1,3-CHD and 1,4-CHD were selected for further study in freezing experiments as described in the next section.

Milestone Contribution (4): "Development of preservation methods to be applied to single cell suspensions and small cell aggregates".

### Cell recovery after freezing

After freezing and thawing the viability of the cell monolayers was markedly decreased compared with the similar groups exposed only to the CPAs without freezing. Fig. 8 shows the relative survival curves for cell monolayers frozen in the respective CPA combinations. Cells frozen in the presence of DMSO, propanediol, or the VS55 combination all showed a "classical" dependence on CPA concentration with an optimum survival in the range of 1-2M CPA. In contrast, cells frozen with formamide alone showed a very low level of recovery over the entire range of concentrations indicating that formamide is a very poor cryoprotectant on its own.

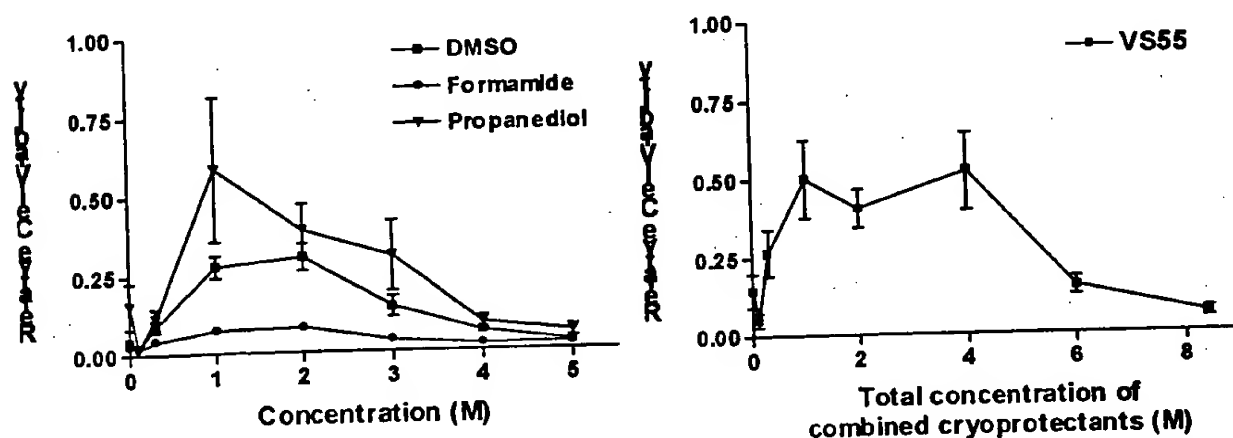


Fig 8. Relative cell viability for AV5 cells after freezing and thawing in either complete VS55 cryoprotectant medium, or in medium containing the individual CPA components of VS55.

In experiments evaluating the effect of selected SIBs, it was determined that these compounds used alone offer virtually no cryoprotection. However, as shown in Fig 9, peak recovery indices at 0.2M 1,3-CHD and 0.3M 1,4-CHD were the same, or in some cases greater than in the comparable freezing experiments carried out in the absence of the SIB molecules (Fig 8). Moreover, it appears that recovery of cells frozen with 1M DMSO was improved when 0.2M 1,3-CHD was incorporated in the solution, but not when 1,4-CHD was used.

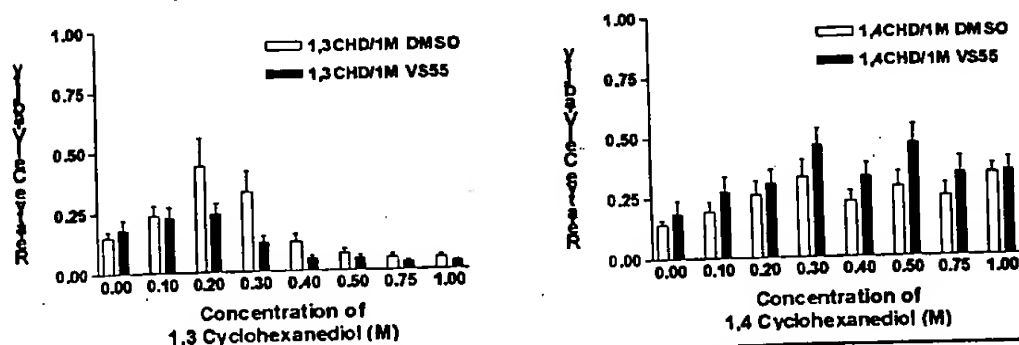


Fig. 9. Effect of the concentration of SIB on the relative viability of AV5 cells frozen in medium containing either DMSO (1M), or VS55 cryoprotectants at a total concentration of 1M.

Two-way analysis of variance (ANOVA) of the data in Fig. 9 showed that cell survival after freezing was highly dependent upon both the composition of the CPA cocktail and the concentration of SIB combined with the CPA. Interaction of these variables was highly significant ( $p < 0.01$ ).

The next step in this study was to test combinations of the CPAs with both synthetic and natural ice-control molecules. For this, AV5 cells were frozen and thawed in the presence of cryoprotectant cocktails containing mixtures of CPAs with SIBs and/or antifreeze proteins according to the schedule summarized in Table 4.

**Table 4. Outline of freezing experiments with ice-control molecules**

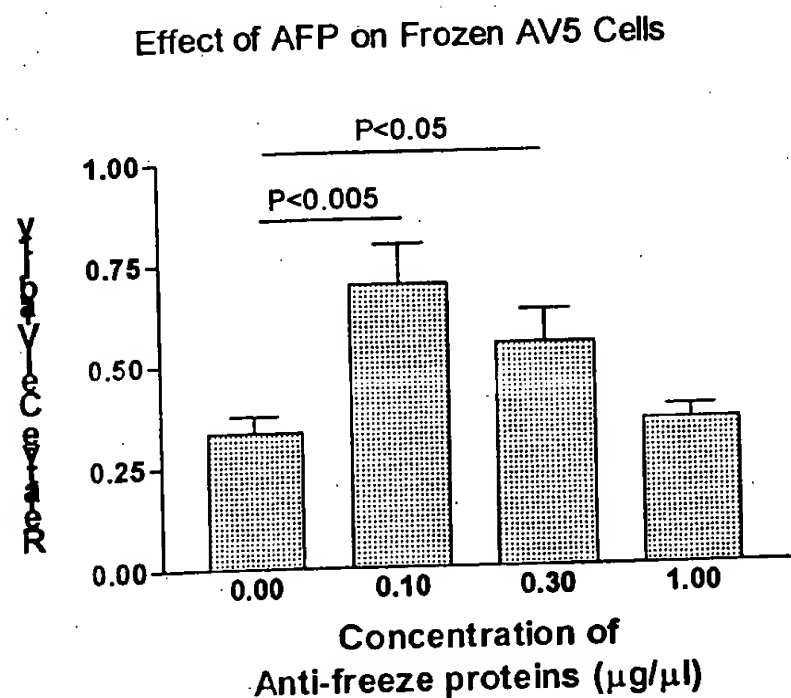
Anti-freeze proteins				
Protein	Concentration range	CPA	N (rep x exp)	
AFPI	0-1 $\mu\text{g}/\mu\text{l}$	1M DMSO	3 rep x 3 exp	
		1M VS55	3 rep x 3 exp	
AFPIII	0-1 $\mu\text{g}/\mu\text{l}$	1M DMSO	3 rep x 3 exp	
		1M VS55	3 rep x 3 exp	
AFGP	0-1 $\mu\text{g}/\mu\text{l}$	1M DMSO	3 rep x 3 exp	
		1M VS55	3 rep x 3 exp	
Anti-freeze proteins with other CPAs and/or SIBs				
Protein	Concentration range	SIB	CPA	N (rep x exp)
AFPI	0-1 $\mu\text{g}/\mu\text{l}$	0.25M 1,3CHD	1M DMSO	3 rep x 3 exp
		0.5M 1,4CHD		
		0.25M 1,3CHD	1M VS55	
		0.5M 1,4CHD		
AFPIII	0-1 $\mu\text{g}/\mu\text{l}$	0.25M 1,3CHD	1M DMSO	3 rep x 3 exp
		0.5M 1,4CHD		
		0.25M 1,3CHD	1M VS55	
		0.5M 1,4CHD		
AFGP	0-1 $\mu\text{g}/\mu\text{l}$	0.25M 1,3CHD	1M DMSO	3 rep x 3 exp
		0.5M 1,4CHD		
		0.25M 1,3CHD	1M VS55	
		0.5M 1,4CHD		

The antifreeze proteins (AFGP, AFPI, and AFPIII), when added to either 1M DMSO solutions, or VS55 (with a total CPA concentration of 1M), did not improve the outcome of the freezing protocol. In some cases the highest concentrations of AFPI and AFP III tested (1mg/ml) was clearly detrimental and reduced the relative cell viability to negligible levels. In contrast, combining the antifreeze proteins with the optimum concentrations of SIB molecules selected from the previous group of experiments, improved the relative cell viability to values that were consistently higher than freezing cells in the same solutions without the SIB molecules. This effect is illustrated in Table 5 for AV5 cells frozen in either DMSO (1M), or VS55 (total CPA = 1M) solutions containing various concentrations of either AFPI, or AFGP. These experiments clearly indicate a synergistic interaction between the antifreeze proteins and the synthetic ice blockers in protecting cells frozen in the presence of classical permeating cryoprotectants such as DMSO and the components of the VS55 solution.

**Table 5. Effect of antifreeze proteins on the relative cell viability of AV5 cells frozen and thawed in the presence of cryoprotectants, SIBs and antifreeze proteins.**

Concn of AFP μg/μl	AFGP/1,4-CHD/DMSO			AFP1/1,4-CHD/VS55			AFGP/1,3-CHD/DMSO			AFP1/1,3-CHD/VS55		
	Survival (%) Mean ± SEM		N	Survival (%) Mean ± SEM		N	Survival (%) Mean ± SEM		N	Survival (%) Mean ± SEM		N
0	26.1	3.8	9	33.2	4.2	9	27.1	3.6	9	31.5	8.1	9
0.1	36.2	4.6	9	69.5	9.8	9	34.4	3.5	9	47.4	10.1	9
0.3	48.6	3.9	9	54.6	8.2	9	38.8	5.3	9	40.9	8.0	9
1.0	42.6	5.4	9	35.5	3.2	9	47.7	6.7	9	10.2	0.8	9

A 2-way ANOVA supports these conclusions by showing that the interaction between the choice of CPA cocktail and the concentration of antifreeze protein for twelve combinations of CPA cocktail and four concentrations of AFP (as outlined in Table 4) was statistically significant in determining the outcome of freezing ( $p < 0.01$ ). The data included in Table 5 is a selection of the complete set of conditions evaluated. The combination of conditions yielding the highest relative survival indices for these cells was AFP1 + 1,4-CHD(0.5M) + VS55(Total CPA=1M). Fig. 10 shows the effect of the concentration of antifreeze protein in this CPA cocktail. The addition of AFP1 led to a statistically significant improvement in cell survival that was clearly dependent on concentration.



**Fig. 10. Effect of concentration of AFP1 on the relative cell viability of AV5 cells frozen and thawed in VS55 (1M) solution containing 1,4-CHD (0.5M). Statistical comparisons between groups were made using unpaired t-tests with the level of significance at 95% confidence.**

We hypothesize that the effects described above are mediated by changes in the structure and amount of ice formation which continues to be a focus in our program as described in the next section.

During the next quarter these studies will be continued by extending the preliminary freezing experiments described previously using the A10 and J15 cells.

Date of Summary: [REDACTED]

**Project Objectives:**

The overall technical goal of this project is the definition and design of conditions for long-term tissue and cell storage that will make it possible for tissue engineered devices to be available both in the United States and worldwide, regardless of environmental conditions.

Specific objectives are:

1. Control of ice crystal formation using a combination of novel synthetic ice blockers (SIBs), naturally occurring antifreeze compounds, ice nucleators and cryoprotectants.
2. Avoidance of ice formation by vitrification.

**Technical Milestones:**

1. Demonstration of non-toxic ice crystal growth control by modulation of ice crystal size in solutions and tissues.
  - (a) Evaluation of the effectiveness of ice growth-inhibiting formulae using physical study methods.
  - (b) Evaluation of the toxicity of ice growth-inhibiting formulae.
  - (c) Evaluation of the results of freezing and thawing vein rings in media designed to contain ice crystal size using *in vitro* and *in vivo* function tests.
2. Creation of new SIB molecular designs with high potential for minimizing ice crystal formation and development of synthesis strategies where necessary.
3. Evaluation of rewarming methods.
  - (a) Identification of the best technique for warming to be deployed for future research and development.
  - (b) Determination of whether or not slower rate resonance radio frequency warming or convection warming is possible with retention of viability in the presence of exogenous agents such as ice nucleating agents.
4. Development of preservation methods to be applied to single cell suspensions and small cell aggregates.
5. Completion of assessment of current first choice baseline vitrification solution consisting of 3.10M DMSO, 3.10M formamide and 2.21M 1,2-propanediol in Euro-Collins Solution. Establishment of an optimal formulation using these 3 reagents in the vascular model.

#### Additional Milestones emerging from Year 1 studies:

6. Development of an alternative vehicle solution for cryoprotectants.
7. Evaluation of alternative vitrification solutions for comparison with the baseline VS55 solution.
8. Physical characterization of new solutions at the microscopic level by cryomicroscopy. This is necessary to complement macroscopic studies in bulk samples that preclude economic use of both AFPs, and newly synthesized SIBs that may be available in limited quantities.
9. Evaluate alternative vitrification solutions using *in vitro* and *in vivo* vascular models compared with baseline techniques established in [REDACTED] to establish an optimized and "user-friendly" technique.
  - Alternative vehicle solution
  - CPA combinations avoiding cytotoxic elements such as formamide.
  - CPA combinations with SIBs
10. Evaluation of optimized technique in allogeneic grafts.

#### Technical Progress and Impact:

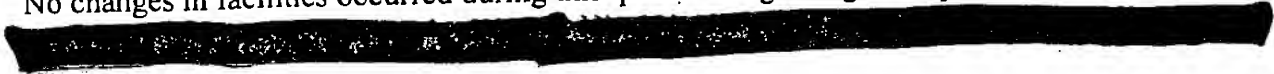
##### Executive Summary

Program momentum has continued during the [REDACTED] Quarter of the Cooperative Agreement. In the last report the excellent *in vitro* function and *in vivo* patency of vitrified vessels were highlighted. Since then the two week analyses of graft explants have been completed. The vitrified grafts had significantly less intimal hyperplasia. Qualitatively the vitrified explants appeared to have denser collagen in the media and a less cellular, thinner adventitia. Longer follow-up of vitrified grafts *in vivo* is required for interpretation of these last observations. The vitrified graft explants did not demonstrate either graft dilation or aneurysmal degeneration nor did they exhibit any other pathology indicative of impending graft failure. *In vitro* cytotoxicity studies have been completed employing three animal sources of vascular cells. The results for each cell strain/line were similar and formamide reduction or replacement was suggested. In contrast cryopreservation experiments with the three cell strain/lines have not been as consistent. New vitrification solution formulation work incorporating information from the cytotoxicity studies has resulted in the discovery of a new formulation (3.0M DMSO, 3.0M propanediol, and 0.5M SIB) with relatively low solute content which exhibits the best ice control of any formulation tested to date. It is anticipated that this new formulation should be compatible with maintenance of tissue viability. Formulation of an improved vehicle solution has also been an important development. Analyses of past and current data have revealed that Euro-Collins solution was having a negative effect on cell survival. The new vehicle solution formulation is superior to Euro-Collins and is at least equivalent to other hypothermic storage solutions employed in the industry. It is likely that this new vehicle solution, Unisol, will have positive effects on cell survival in both molecular ice control and vitrification procedures. Histology and freeze

substitution studies demonstrated both good cell preservation and modification of ice morphology in the presence of 0.5M 1,3-cyclohexanediol and 1M DMSO. Further morphometric analyses and viability testing of vein rings preserved with this ice control formulation will be performed during the coming quarter.

#### *Facility & Personnel*

No changes in facilities occurred during this quarter. Regarding staff, position #7 (cryophysicist)



#### *Current Personnel.*

Milestone Contribution (1a): "Evaluation of the effectiveness of ice growth-inhibiting formulae using physical study methods"; (2) "Creation of new SIB molecular designs with high potential for minimizing ice crystal formation and development of synthesis strategies where necessary"; and (7) "Evaluate alternative vitrification solutions using lower total concentrations of CPAs than baseline VS55"

## Ice Crystal Growth Studies

In the previous report we described the profound effect that our current range of synthetic ice blocker (SIB) compounds had on both the formation of ice during the slow cooling (1-3°C/min) of bulk samples of cryoprotectant solutions, and on the rates of ice crystal growth. Two compounds in particular, 1,3-cyclohexanediol (1,3-CHD) and 1,4-cyclohexanedione (1,4-CHD) were selected for further study based upon their robust effect in controlling ice formation in these physical studies. Moreover, the importance of developing new combinations of cryoprotectants with a total solute concentration lower than the baseline vitrification solution, VS55, was emphasized on the grounds of reducing cytotoxicity in the biological model systems. We have proceeded therefore, with the plan specified at the end of the previous report, to investigate the effect of the SIBs in a mixture of dimethyl sulfoxide (DMSO) and propanediol without formamide, which we have found to be especially toxic to cells. The focus during this quarter has been to combine these SIBs, either alone or in combination, with 3.0 mols/l each of DMSO and propanediol (a new solution designated DP6), since we have shown previously (see p55-56 Y1Q4 report) that a solution comprising 3.0M DMSO and 3.4M propanediol (F2) had similar ice crystal growth rates compared with the baseline VS55 vitrification solution. Furthermore, 6% (~0.5M) 1,3-CHD was highly effective in reducing ice crystal growth in V49 (7.5M CPA) to rates significantly lower than either of the aforementioned solutions. DP6 was formulated therefore, to contain a total CPA concentration = 6.0M; that is, lower than any of the solutions tested thus far, and supplemented with 6% SIBs which is known to have a marked effect on ice crystal growth during slow cooling.

Table 1 shows an update of the bulk phase ice crystallization measurements in various solutions and also includes the preliminary data for DP6 containing SIBs. It can be seen that ice crystal formation in DP6+6%1,3-CHD was undetectable, and was negligible in DP6 containing 1,4-CHD alone, or in combination with 1,3-CHD. Fig1. illustrates these effects by showing photographs of the bulk cooling cassettes containing various solutions during slow cooling (1-3°C/min) to -100°C. The digital images in Fig 1. are representative of those used to quantify ice formation in these bulk samples using an image analysis technique described previously.

**Table 1: Bulk Phase Ice Crystallization Measurements from Image Analysis**

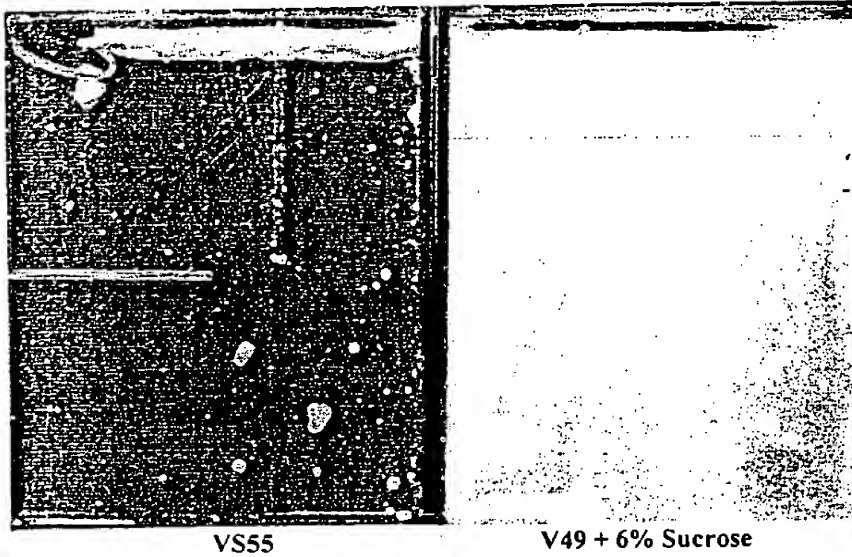
Cryoprotectant Solution	Ice Crystal #	Total Area %	Cooling rate °C/min
V49 (7.5M CPAs)	Indefinite	100	2.4±0.4 (4)
VS55 (8.4M CPAs)	49.6±6.4 (32)	1.31 ± 0.12 (32)	1.5±0.1 (32)
V49 + NaCl	609 ± 104 (3)	22.2 ± 1.0 (3)	1.2±0.1 (3)
V49 + Sucrose	5.3 ± 2 (3)	99.8 ± 0.1 (3)	1.2±0.2 (3)
V49 + 1,2-Cyclohexanedione (1,2 CHO)	107 ± 23 (2)	3.6 ± 0.2 (2)	1.6±0 (2)
V49 + 1,3-Cyclohexanedione (1,3 CHO)	12.5 ± 8.5 (2)	0.32 ± 0.27 (2)	1.1±0.3 (2)
V49 + 1,2-Cyclohexanediol (1,2 CHD)	108 ± 55 (3)	2.16 ± 0.7 (3)	1.6±0 (2)
V49 + 1,3-Cyclohexanediol (1,3 CHD)	173 ± 76 (4)	2.27 ± 0.7 (4)	2.1±0.4 (4)
V49 + 1,4-Cyclohexanediol (1,4 CHD)	107 ± 66 (5)	1.68 ± 0.55 (5)	1.3±0.2 (5)
V49 + 1,3 and 1,4-Cyclohexanediol	46.8±16.4 (4)	0.73±0.2 (4)	1.4±0.1 (4)
DP6 + 1,3- Cyclohexanediol (1,3 CHD)	0±0 (5)	0±0 (5)	1.2±0.1 (5)
DP6 + 1,4-Cyclohexanediol ( 1,4 CHD)	10±9 (2)	0.3±0.3 (2)	1.2±0.1 (2)
DP6 + 1,3 and 1,4-Cyclohexanediol	2	0.01	1.1

Values are the Mean ± SEM (n).

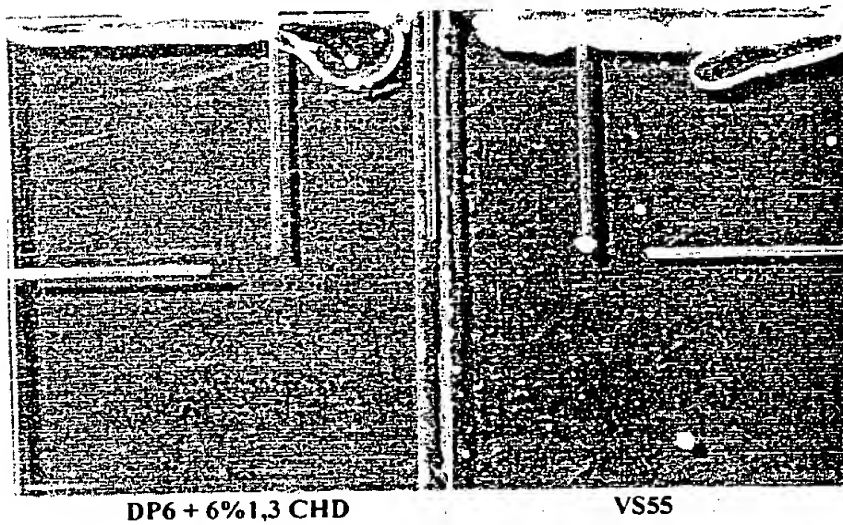
### Ice Crystal Growth Kinetics:

The importance of measuring ice crystal growth kinetics in assessing the efficacy of ice control molecules has been emphasized in previous reports. We have shown that 6% (0.5M)1,3-CHD dramatically reduced the peak rate of ice crystal growth in V49 (total CPA concentration = 7.5M) solution by a factor of twenty in the range -45°to-55°C. Fig 2. Shows the results of recent experiments in which SIBs were added to the new DP6 solution containing a final solute concentration of 6.5M. For comparison ice crystal growth kinetic data for the DP6 solutions is plotted on the same graphs as shown previously for other solutions (Fig 3. Page 55 report). In particular, Fig 2A shows the robust effect of 1,3-CHD in reducing the peak growth rates of DP6 to <50µm/min over the temperature range of maximum growth rate in the F1and F2 solutions. Furthermore, the total concentration of DMSO and propanediol in DP6 is lower than either F1(comprising DMSO(3.0M) and propanediol (3.2M)), or F2 (containing 3.0M DMSO and 3.4M propanediol). Fig 2B. illustrates the effect of combining 1,3 and 1,4-CHD in DP6 when compared with the baseline vitrification solution VS55, or V49 containing 6% sucrose. The latter is used as a control solute having a high colligative function and also considered to be a good glass-forming agent.

Temperature = -100.1, Cooling Rate = 1.11°C/min



Temperature = -101.8, Cooling Rate = 1.3°C/min



Temperature = -109.8, Cooling Rate = 1.5°C/min

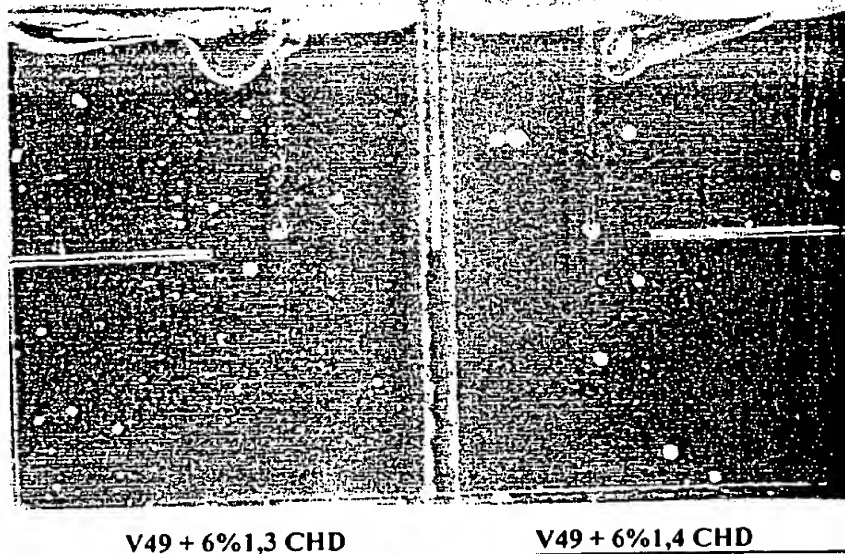


Fig 1. Photographs of various solutions cooled to below -100°C at the rates indicated in a dual chamber transparent cassette containing 75ml of each solution. Ice is apparent as white areas contrasted against a dark background. Note the extensive ice in V49+6% sucrose and no detectable ice in DP6 +6% 1,3-CHD.

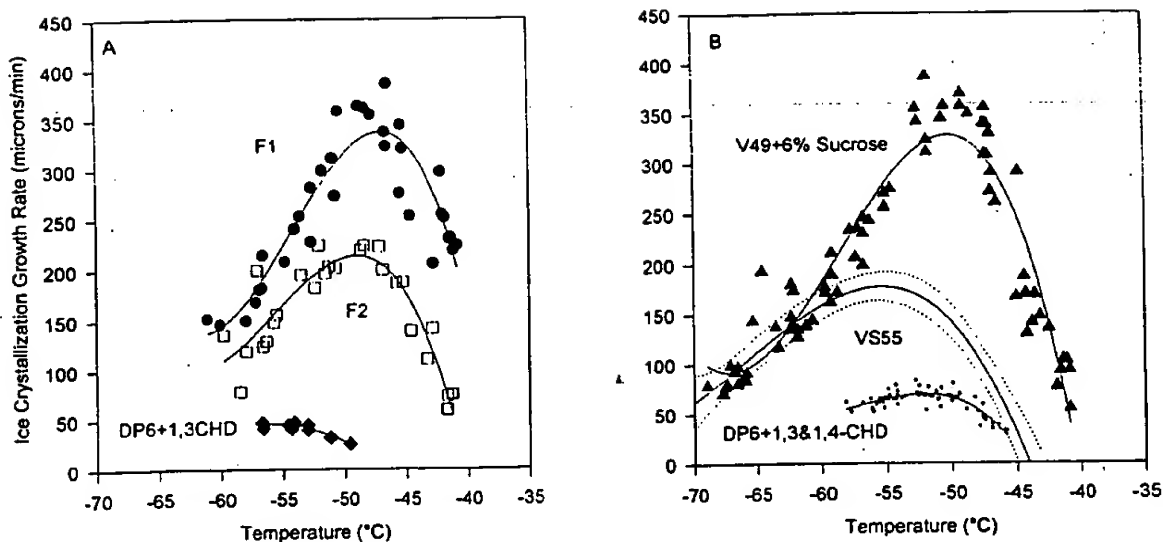


Figure 2. Ice crystal growth kinetics for new alternative vitrification solutions compared with VS55 baseline solution. F1 = 3M DMSO + 3.2M Propanediol; F2 = 3M DMSO + 3.4M Propanediol; DP6 = 3M DMSO + 3M Propanediol. The graphs show 3<sup>rd</sup> order regression plots (solid lines) fitted to the data in each case.

The data for DP6+combined SIBs is also plotted on an expanded scale in Fig 3 showing a strong control of ice crystal growth in the temperature range of maximum growth in VS55 and F2. Moreover, the regression curve overlays that of V49 + 0.5M1,3-CHD which has a 23% higher total concentration of solutes. We already know from our cell studies that both DMSO and Propanediol are well tolerated at a concentration of 3M. We anticipate therefore, that the DP6 solution, containing 3 mols/l of each CPA plus 0.5M SIB will provide an excellent ice-control medium that should be compatible with maintenance of cell viability.

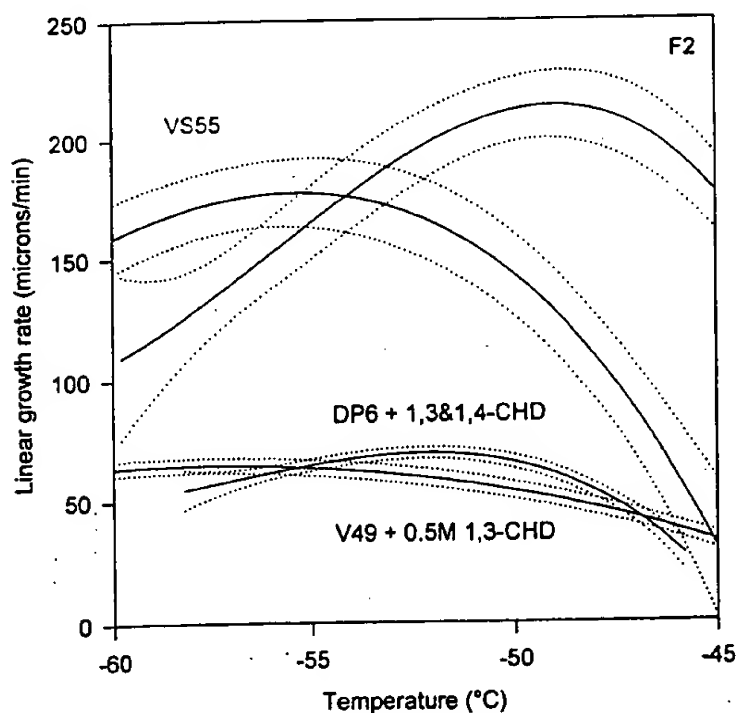


Fig. 3. Comparative kinetics of ice crystal growth in solutions designed to vitrify, or inhibit ice nucleation and growth. Solution designations are defined in the text. The graphs are plotted as 3<sup>rd</sup> order regression curves with 95% confidence limits shown in each case and the data points are omitted for clarity.

### Microscopic Observations of Frozen Tissues:

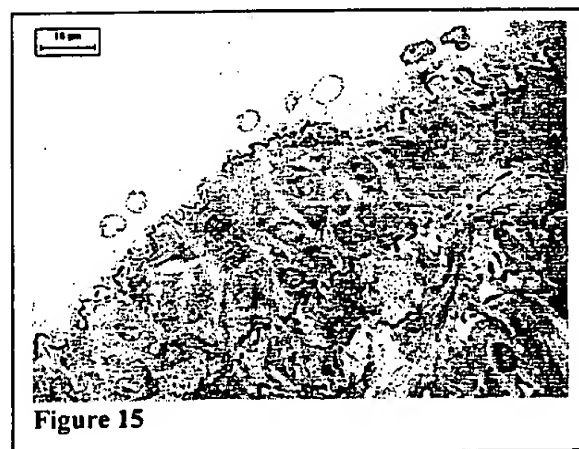
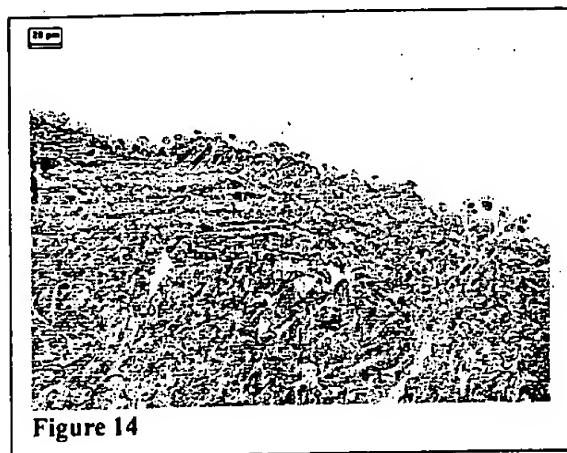
Jugular vein rings were frozen in one of the four solutions listed in Table 4 using a standard protocol of controlled cooling ( $1^{\circ}\text{C}/\text{min}$ ) to  $-80^{\circ}\text{C}$ , followed by storage in the vapor phase of liquid nitrogen. (The selection of solutions for these morphology studies was based largely upon the results of our previous physical studies with the SIBs, and subsequent cytotoxicity experiments with isolated cells). Some samples were thawed and processed for light microscopy after removal of the CPAs; other samples were freeze-substituted at  $-90^{\circ}\text{C}$  using the methanol technique we have described in previous reports. Sections of  $0.75\mu\text{m}$  thickness were obtained and stained with toluidine blue. This study was conducted on a double-blind basis in that our microscopist, Mr Lightfoot, was blinded to the designation of the code in Table 4 until the completion of the study and his observations had been recorded. The following observations were extracted from his report:

**Table 4**  
Designation of Solutions used to freeze vein rings

- A: EuroCollins (EC) Solution
- B: 1M DMSO in EuroCollins
- C: 0.5M 1,3-Cyclohexanediol + 1M DMSO in EC
- D: 0.5M 1,4 Cyclohexanediol + 1M DMSO in EC

#### *Morphology of frozen-thawed tissue*

Figure 14 & 15 show tissues treated with solution A. The three regions of the vein are clearly discernible. The intima exhibits a swelling of the endothelial cells and there are small vacuoles also visible within this region. The smooth muscles bundles within the media have been rearranged and appear perpendicular to the plane of section.



When tissues were frozen Solution B (Figures 16 & 17) there was extreme swelling of the endothelial cells and subsequent sloughing of cells into the lumen of the blood vessel. Some of the cells appear to be enucleated and considerable vacuolization can be seen throughout the intima. Again, the smooth muscle cells have a rearranged profile.

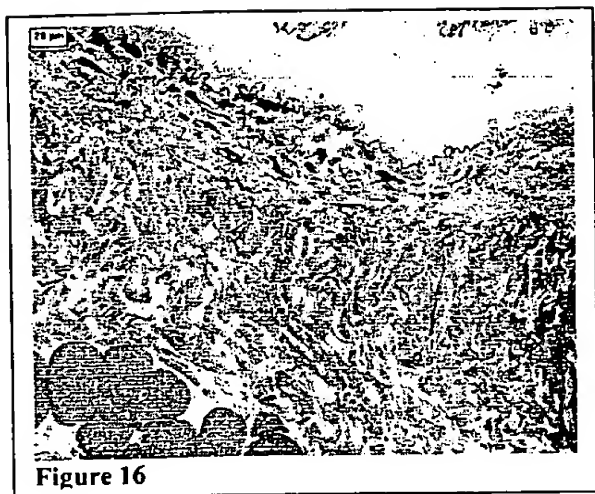


Figure 16



Figure 17

Cryopreservation in Solution C appeared to cause the least amount of cellular damage within the Tunica intima and media compared to the other three solutions used in this study. At low magnification (Fig. 18) the endothelial cell lining appears to be more intact with little evidence of cell swelling and/or vacuolization. At higher magnification (Fig.19) the endothelial cells show a well-defined nucleus and the smooth muscle cells are perpendicular to the plane of section.

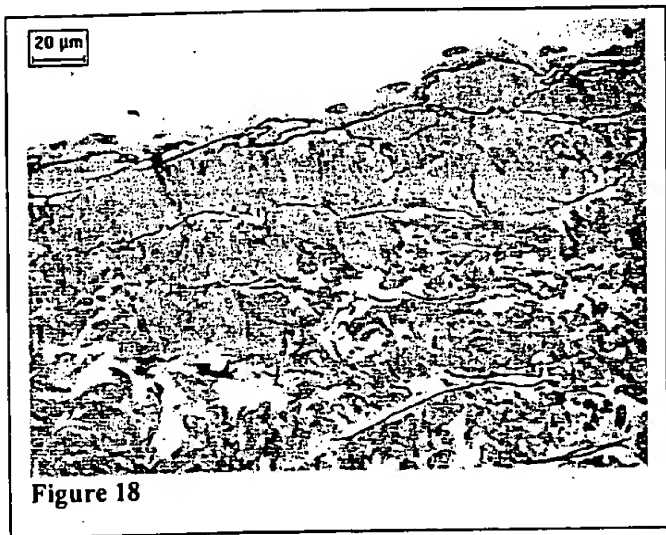


Figure 18

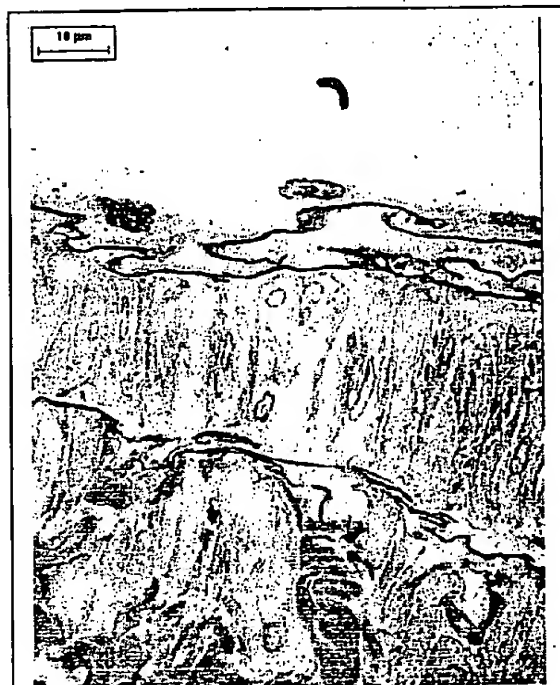
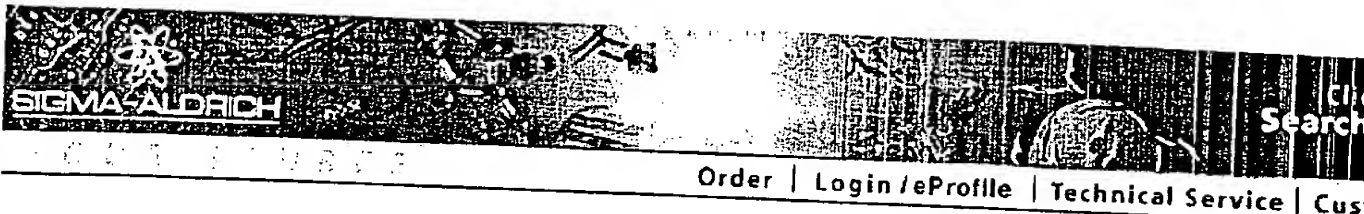


Figure 19

Exhibit

B

Product Number: **C10,110-9**Product Name: **1,3-Cyclohexanediol (cis+trans)**
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**Synonyms:** Hexahydroresorcinol**MDL number:** MFCD00039458**Molecular Formula:** C<sub>6</sub>H<sub>12</sub>O<sub>2</sub>**Molecular Weight:** 116.2**CAS Number:** 504-01-8**MDL Number:** MFCD00039458**Assay:** 98%**EC Number:** 2079795**Beilstein Index:** Beil.6,IV,5208**Comments:**

98%

Extended specifications	
bp	246-247 °C/760 mm Hg (lit.)
Fp	>230 °F

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